JC09 Rec'd PCT/PTO 2 7 JUN 2001

FORM-PTO-1390 (Rev. 12-29-99) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE **TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371**	ATTORNEY'S DOCKET NUMBER 003300-790 U S APPLICATION NO (If known, see 37 C F.R. 1 5) 09/869269

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/SE99/02446	22 December 1999	29 December 1998

TITLE OF INVENTION
MODIFICATION OF INTERFERON ALPHA PRODUCTION

APPLICANT(S) FOR DO/EO/US
ÅSA BERGLUND

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and the PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). (Signed Declaration will follow).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:

A certified of Swedish Application No. 9804583-4, filed 29 December 1998, was submitted during the international phase of prosecution. Thus the claim for priority has been perfected.

JC18 Rec'd PCT/PTO 27 JUN 2001

U.S. APPLICATION NO. (If known,/ see 37 C.F.R. 1 50)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/869269	PCT/SE99/02446	003300-790

17. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO .... $1,000.00 (960) International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO .... $860.00 (970) International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO .... $710.00 (958) International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) .... $690.00 (956) International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) $100.00 (962)		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 1,000.00	
Surcharge of **$130.00 (154)** for furnishing the oath or declaration later than 20 ☐ 30 ☐ months from the earliest claimed priority date (37 CFR 1.492(e)).	$ --	

Claims	Number Filed	Number Extra	Rate		
Total Claims	20 -20 =	--	X$18.00 (966)	$ --	
Independent Claims	1 -3 =	--	X$80.00 (964)	$ --	
Multiple dependent claim(s) (if applicable)			+ $270.00 (968)	$ --	

	CALCULATIONS	PTO USE ONLY
TOTAL OF ABOVE CALCULATIONS =	$ 1,000.00	
Reduction for 1/2 for filing by small entity, if applicable (see below).	$ 500.00	-
SUBTOTAL =	$ 500.00	
Processing fee of **$130.00 (156)** for furnishing the English translation later than 20 ☐ 30 ☐ months from the earliest claimed priority date (37 CFR 1.492(f)). +	$ --	
TOTAL NATIONAL FEE =	$ 500.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00 (581)** per property +	$ --	
TOTAL FEES ENCLOSED =	$ 500.00	
	Amount to be: refunded	$
	charged	$

a. ☒ Small entity status is hereby claimed.

b. ☒ A check in the amount of $ 500.00 to cover the above fees is enclosed.

c. ☐ Please charge my Deposit Account No. 02-4800 in the amount of $______ to cover the above fees. A duplicate copy of this sheet is enclosed.

d. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-4800. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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Filed: June 27, 2001

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22,030
REGISTRATION NUMBER

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JC18 Rec'd PCT/PTO 27 JUN 2001

Patent
Attorney's Docket No. 003300-790

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	)	Box: PCT
	)	
ÅSA BERGLUND	)	Attention: DO/EO/US
	)	
Application No.: (unassigned)	)	Group Art Unit: (unassigned)
	)	
Filed: June 27, 2001	)	Examiner: (unassigned)
	)	
For: MODIFICATION OF INTERFERON ALPHA PRODUCTION	)	

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

This is a national phase filing of International Application No. PCT/SE99/02446, filed December 22, 1999.

It is contemplated that this Application be prosecuted in the United States while using Specification Page Nos. 13 and 14 and Claims 1 to 9 that were submitted on January 24, 2001 during the international stage of prosecution as further amended herein.

Please amend the above-identified Application as indicated.

IN THE ABSTRACT:

Please add the Abstract of the Disclosure that is provided on a separate sheet.

IN THE CLAIMS:

Kindly replace Claims 3 to 8, and add new Claims 10 to 20 as follows.

3. (Amended) A process according to claim 1, characterized in that the enhancing agent is added at the same time or up to 4 hours after the virus induction.

4. (Amended) A process according to claim 1, characterized in that the virus is Sendai virus.

5. (Amended) A process according to claim 1, characterized in that the enhancing agent is theophylline.

6. (Amended) A process according to claim 1, characterized in that the enhancing agent is 2-amino-5-bromo-6-methyl-4-pyrimidinol.

7. (Amended) A process according to claim 1, characterized in that the enhancing agent is thymine.

8. (Amended) A process according to claim 1, characterized in that the organic solvent is any of acetone, 2-butanone, 1,3-dimethyl-2-imidazolidinone, dimethylsulfoxide, N-ethyl-2-pyrrolidinone, 4-methyl-2- pentanone, N-methyl-2-pyrrolidinone, 2-pyrrolidinone, tetramethylene sulfoxide and N,N-dimethylacetamide.

10. (New) A process according to claim 2, characterized in that the enhancing agent is added at the same time or up to 4 hours after the virus induction.

11. (New) A process according to claim 2, characterized in that the virus is Sendai virus.

12. (New) A process according to claim 3, characterized in that the virus is Sendai virus.

13. (New) A process according to claim 10, characterized in that the virus is Sendai virus.

14. (New) A process according to claim 2, characterized in that the enhancing agent is theophylline.

15. (New) A process according to claim 3, characterized in that the enhancing agent is theophylline.

16. (New) A process according to claim 4, characterized in that the enhancing agent is theophylline.

17. (New) A process according to claim 2, characterized in that the enhancing agent is 2-amino-5-bromo-6-methyl-4-pyrimidinol.

18. (New) A process according to claim 3, characterized in that the enhancing agent is 2-amino-5-bromo-6-methyl-4-pyrimidinol.

19. (New) A process according to claim 2, characterized in that the enhancing agent is thymine.

20. (New) A process according to claim 2, characterized in that the organic solvent is any of acetone, 2-butanone, 1,3-dimethyl-2-imidazolidinone, dimethylsulfoxide, N-ethyl-2-pyrrolidinone, 4-methyl-2- pentanone, N-methyl-2-pyrrolidinone, 2-pyrrolidinone, tetramethylene sulfoxide and N,N-dimethylacetamide.

REMARKS

The present amendment is intended to eliminate the use of multiple dependency.

The examination and allowance of the application are respectfully requested.

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS, L.L.P.

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Abstract of the Disclosure

A process for the production of α-interferon comprising the steps: i) inducing of human leukocytes by means of a virus; ii) treating the leukocytes with an enhancing agent selected from: a) Xanthine, pyrimidinol and pyrimidinone or derivatives of anyone thereof, such as theophylline, 2-amino-5-bromo-6-methyl-4 pyriminidol or thymine; b) an organic solvent selected from the group consisting of non-aromatic ketones, aliphatic or cyclic amides, alkylated aliphatic or cyclic urea derivatives and aliphatic or cyclic sulfoxides, such as N-methyl-2-pyrrolidinone, acetone, 2-butanone, 1,3-dimethyl-2-imidazolidinone, dimethylsulfoxide, 4-methyl-2-pentanone-N-ethyl-2-pyrrolidinone, 2-pyrrolidinone, tetramethylene sulfoxide or N,N-dimethylacetamide; or a combination of the compounds from a) with an organic solvent from b).

Attachment to Preliminary Amendment dated June 27, 2001

Marked-up Claims 3 to 8

3. (Amended) A process according to [any one of claims 1 and 2] claim 1, characterized in that the enhancing agent is added at the same time or up to 4 hours after the virus induction.

4. (Amended) A process according to [any one of claims 1 - 3] claim 1, characterized in that the virus is Sendai virus.

5. (Amended) A process according to [any one of claims 1 - 4] claim 1, characterized in that the enhancing agent is theophylline.

6. (Amended) A process according to [any one of claims 1 - 4] claim 1, characterized in that the enhancing agent is 2-amino-5-bromo-6-methyl-4-pyrimidinol.

7. (Amended) A process according to [any one of claims 1 - 4] claim 1, characterized in that the enhancing agent is thymine.

Attachment to Preliminary Amendment dated June 27, 2001

Marked-up Claims 3 to 8

8. (Amended) A process according to [any one of the preceding claims] claim 1, characterized in that the organic solvent is any of acetone, 2-butanone, 1,3-dimethyl-2-imidazolidinone, dimethylsulfoxide, N-ethyl-2-pyrrolidinone, 4-methyl-2-pentanone, N-methyl-2-pyrrolidinone, 2-pyrrolidinone, tetramethylene sulfoxide and N,N-dimethylacetamide.

MODIFICATION OF INTERFERON ALPHA PRODUCTION

Field of invention

The present invention is directed to a process for the production of α-interferon in human leukocytes induced by virus. More particularly the invention relates to a process of production of α-interferon in which process the leukocytes are treated with an enhancing agent.

Background of the invention

The Interferons constitutes a family of proteins characterized by their non-specific antiviral and antiproliferative activity, a property that has made them useful as antiviral and anticancer drugs. Interferons are produced and released by animal cells upon exposure to a variety of inducing agents, the most potent of which are viruses. They are classified into three types: α-Interferon, β-Interferon, and γ-Interferon, based on serological and structural relations. The use of interferons as therapeutic drugs dates back to the 1970's. Although all three types of interferons have been subject to evaluation, α-interferon has become most widely used for therapeutic purposes. Among the interferons of human origin, the α-interferons are divided into several *subtypes*, which are either encoded by different gene loci or alleles of those, while there is only one subtype each of human β- and γ-interferon. The function of each subtype is still not clear, and the molecular or cellular targets of their antiviral and antineoplastic activities is thus not fully investigated. However, some *in vitro* studies have shown a larger biological effect when a mixture of different subtypes was used compared to using a single subtype only (Fan, S.X, Skillman, D.R, Liao, M-J, Testa, D. and Meltzer,

M.S. **(1993)** *AIDS Res. And Human Retrovir.* ***9,*** 1115-1122, Heim, A., Brehm, C., Stille-Siegener, M., Müller, G., Hake, S, Kandolf, R. and Figulla, H-R. **(1995)** *J. Mol. Cell Cardiol..* ***27,*** 2199-2208).

There are presently three major methods for industry-scale production of α-interferon, all with fundamental differences in the cell system used. In the procaryotic systems, the gene coding for a single subtype, almost exclusively interferon α2, has been transferred to *Escherichia coli*, whereby this protein is expressed by the bacteria and subsequently harvested (Wessmann,C., Hagata,S., Boll,W., et al **(1982)** *Phil. Trans. Royal Soc. London, Series B: Biol. Sci. 299* 7-28). This process for the production of α-interferon is referred to herein as "recombinant interferon". The bacterial cultures can be used for producing α-interferon at high efficiency, leading to an economically advantageous alternative to cells of human origin. However, only one single subtype is produced and the proteins are not modified (e.g. glycosylated) in contrast to interferon α2 that is produced in eukaryotic cells. A clinical drawback encountered with the recombinant α-interferon products is their tendency to induce antibodies against α-interferon in some patients. These neutralising antibodies have in several publications been shown to negatively affect the therapeutic treatment with recombinant α-interferon (Antonelli, G., Simeoni, E., Currenti, M., DePisa, F., Colizzi, V., Pistello, M., and Dianzani, F., **(1997)** *Biother* **10**, 7-14, Öberg, K. and Alm, G. **(1997)** *Biother* **10**, 1-5).

Alternatively α-interferon can be produced in human cells, either from established cell lines which are grown *in vitro*, or from primary cells, e.g., from peripheral leukocytes obtained as by-products from donated blood. In these case a mixture of α-interferon subtypes is

obtained, although different cell sources produce a different subtype pattern. (Goren, T., Fischer, D.G. and Rubinstein M. **(1986)** *J. Interferon* Res. 6, 323-329. Established cell lines are clones derived from human tumors or from cells that have been immortalised, e.g., by the treatment with Epstein-Barr virus. These cells divide and grow indefinitely, in suitable media and under appropriate conditions. In contrast, interferon-producing primary cells such as leukocytes do not divide and have a finite life span. Such cells are consequently in limited supply, and their availability is a limiting factor for large scale production of native leukocyte α-interferon. Means for increasing the yield in the production process are therefore necessary.

Eucaryotic cell systems produce very little or no α-interferon spontaneously. The use of an "*inducer*" is therefore needed to initiate the production of α-interferon by the cells. Consequently, a large number of factors have been reported to initiate the production of α-interferon in various *in vitro* cell systems or *in vivo*. The most common inducers are different viruses, but synthetic organic substances have also been shown to act as inducers for the production of α-interferon. Some examples are leu-enkephalin and naloxone *in vivo* in mice (Gabrilovac, J., Ikic-Sutlic, M., Knezevic, N. and Poljak, L. **(1996)** *Res. Exp. Med.* ***196,*** 137-144), neovir *in vivo* in mice (Mezentseva M., Narovlyansky, A., Kondratieva, T and Ershov, F. **(1997)**. *J.Interferon Res.* *17, Suppl.2,* S94. Abstract), dipyridamole *in vivo* in humans (Galabov A.S. and Mastikova M. **(1984)** *Biomed. Pharmacoth.* ***38***, 412-413), 2-amino-5-bromo-6-methyl-4-pyrimidinol (2-ABMP) and its derivatives *in vivo* in humans(US Patent No 3,932,617, Stringfellow, D.A., Vanderberg, H.C. and Weed, S.D. **(1980)** *J. Interferon Res.* ***1,*** 1-14) and antraquinone derivatives *in vivo* in a variety of species (US patent 4,027,021). Ethanol is

reported to induce β-interferon production in Madin-Darby bovine kidney (MDBK) cells in the absence of other inducers (Chelbi-Alix, M.K. and Chousterman, S. **(1992)** *J. Biol. Chem.* ***267,*** 1741-1745).

There are some examples where the production of α-interferon has been increased by ancillary reagents, termed herein as "*enhancers*", i.e., compound(s) which is/are capable of increasing the production of α-interferon in cells activated by an inducing agent, but does not itself induce production of α -interferon. Characteristic of an enhancer is that it can be added either before the agent that induces α-interferon, or after the induction has taken place. Substances that are reported as enhancers of interferon production in human cells are, e.g., the calmodulin inhibitor trifluoperazine used on fibroblasts (Lin, H-Y. and Thacore, H.R. **(1990)** *J. Interferon Res.* ***10,*** 375-378), dexamethasone used as a stimulator on a cell line of lymphoblastoid origin (US patent 4,266,024) and sodium butyrate, also used as a stimulant in a lymphoblastoid cell line (EP 0097 353, EP 0000520). Furthermore, it has been reported that the purine derivative theophylline acts as an enhancer and increases the yield of α-interferon from mouse Lpa cells induced by poly I:C (Zahorska, R., Korbecki, M., and Barciszewski, J. **(1995)** *Arch. Immunol. Ther. Exp.* ***43***, 43-46), and the use of some synthetic organic compounds, preferably tetramethylurea (TMU) (European Patent No 0 097 353), or dimethylsulfoxide (DMSO) (US Patent No 4,266,024) has been shown to increase the α-interferon yield from a lymphoblastoid cell line treated with an inducer.

In the work leading to the present invention some of the substances which has been reported as inducers or enhancers when used in vivo or on fibroblasts and on a cell line of lymphoblastoid origin has been tested on

human leukocytes, with the aim to increase the α-interferon production, both before or after induction with Sendai virus, but without any positive result on the yield of α-interferon. In some experiments even a decrease in α-interferon production from human leukocytes was observed when adding compounds known as enhancers in cell lines. For instance, pre-treatment with sodium butyrate has been described as a stimulant that leads to an increased yield of α-interferon in a lymphoblastoid cell line (European Patent No 0 097 353; European Patent No 0 000 520). In the present work it was found that pre-treatment of primary leukocytes with sodium butyrate according to a similar procedure led to a *decrease* in the yield of α-interferons, which points at fundamental differences between cell lines and primary cells as production systems for α-interferon proteins.

It is accordingly an object of the present invention to provide an improved process for production of α-interferon in human leukocytes induced by virus.

<u>Summary of the invention</u>

The object of the invention is obtained by the process for the production of α-interferon as claimed in the claims.

According to the invention there is provided a process for the production of α-interferon comprising the steps:

i) inducing of human leukocytes by means of a virus,

ii) treating the leukocytes with an enhancing agent selected from

a) Xanthine, pyrimidinol and pyrimidinone or derivatives of anyone thereof;

b) an organic solvent selected from the group consisting of non-aromatic ketones, aliphatic or cyclic amides,

alkylated aliphatic or cyclic urea derivatives and aliphatic or cyclic sulfoxides; or a combination of the compounds from a) with an organic solvent from b).

According to the present invention, it was surprisingly found that the addition of the compounds and/or an organic solvent as indicated above, increases the amount of virus induced α-interferon in leukocytes. Considering the different species in the case of the mouse Lpa cells and the different cell source in the case of the lymphoblastoid cell line, and considering the lack of consistency between the different cell systems in the experiments described above, it was surprisingly found that theophylline as well as a solvent such as dimethylsulfoxide enhanced the production of α-interferon in virus induced primary leukocytes. Also, the pyrimidine derivative 2-amino-5-bromo-6-methyl-4-pyrimidinol (2-ABMP), was found to act as an enhancing agent of the α-interferon production according to the present invention using virus induced primary leukocytes. Since 2-ABMP did not induce α-interferon in the absence of a viral inducer using the experimental system described in this invention, the mechanism for enhancement is obviously different for the *in vivo* α-interferon induction described in the literature, as compared to the α-interferon production enhancing effect of this substance observed in the present invention. This further strengthens the evidence that different mechanisms are in effect in different human cell systems, and indicates the unpredictiveness in working with additives to cellular systems or organisms.

As mentioned above several agents have been used as inducers and enhancers in both in vivo systems and in different cell culture systems. Since the data in the literature in many cases deviated from the findings in the present invention e.g. the inability for 2-ABMP to

act as an interferon inducer in the cell system according to the invention, several unique properties of the cellular system in this invention should be pointed out. Primary cells from peripheral blood represent a resting cell population in contrast to all the other cellular systems described for interferon production. The cells are not activated or immortalised by virus, as is the case for Burkitt's lymphoma cells or so called lyphoblastoid cells. Nor are they tumor cell lines with their inherent genomic instability and dysregulated gene expression. It has been shown that within a mixture of leukocytes derived from human blood the monocytes are the main producers of α-interferon after Sendai virus treatment (Sandberg, K., Matsson, P. And Alm, G. **(1990)** *J. Immunology* **145**, 1015-1020). Thus, the cells used in this invention differ from the established cell lines used for α-interferon production with respect to growth state, neoplastic potential, presence of viral genome and cell type. The effects of the tested factors are therefore a priori not expected to be the same as for other cell types, with differences in differentiation, metabolic state or interference from viral genomes.

Detailed description of the invention

According to the present invention it was found that addition of enhancing agents in the form of xanthine, pyrimidinol and pyrimidinone or derivatives of anyone thereof and/or various organic solvents significantly increases the interferon production in human leukocytes induced by virus. The enhancing agent can increase the interferon yield either alone or in a combination of an organic solvent and the mentioned compounds. At certain conditions the effect of combining these enhancing agents is synergistic, showing a larger effect than the one obtained using the substances separately.

The xanthine derivatives that can be used according to the invention are xanthine with aliphatic and/or aromatic substituens, such as theophylline, theobromine, enprophylline, hypoxanthine and 8-phenyltheophylline. Theophylline is a preferred enhancing agent. Pyrimidinol derivatives that can be used are for example 2-amino-5-bromo-6-methyl-pyrimidinol and 2-amino-6-methyl-4-pyrimidinol with 2-amino-5-bromo-6-methyl-pyrimidinol as a preferred embodiment. A preferred pyrimidinone derivative is thymine.

The organic solvents that can be used according to the invention are non-aromatic ketones, aliphatic or cyclic amides, alkylated aliphatic or cyclic urea derivatives and aliphatic or cyclic sulfoxides. As preferred solvents can be mentioned acetone, 2-butanone, 1,3-dimethyl-2-imidazolidinone, dimethylsulfoxide, N-ethyl-2-pyrrolidinone, 4-methyl-2-pentanone, N-methyl-2-pyrrolidinone (NMP), 2-pyrrolidinone, tetramethylene sulfoxide, N,N-dimethylacetamide, 2-pyrrolidinone. The most preferred compounds presented under item a) above with respect to interferon production is theophylline and 2-ABMP. The most preferred solvent is NMP.

The virus used can be any virus, but the preferred virus is Sendai virus, the most potent inducer of interferon-α for purified human leukocytes.

Leukocyte purification, incubation of the cells and induction by virus are mainly performed according to the original method of Cantell et al. (Cantell, K., Hirvonen, S., Kauppinen, H-L. and Myllylä, G. (1981) Methods in Enzymology 78, 29-38).

The human leukocytes used according to the invention are prepared from buffy coats by an initial centrifugation to

fractionate the components, followed by sequential removal of the plasma layer and of the leukocyte fraction by suction. Residual red blood cells contaminating the leukocyte fraction are lysed twice with 2 - 4 volumes of cold 0.83 % ammonium chloride and each lysis step is followed by centrifugation to collect the cells. The final leukocyte fraction is resuspended in a basal medium, e.g. EMEM (Eagle's minimum essential medium) supplemented with polyethylene glycol- (PEG)-precipitated human plasma. PEG-precipitated plasma is prepared by adding PEG 6000 to a final concentration of 6 % (w/w) to human plasma. After precipitation for 3-5 days in a cold room the supernatant is removed and stored frozen until used. The temperature of the incubation medium is set to 36 -37°C and continuous stirring is used. Human PEG-precipitated plasma is added to a concentration of 1 - 5 % (v/v), preferably 4 % (v/v). A low amount of α-interferon (normally 100 IU/ml) is added to the incubation medium as a priming step, but other concentrations, up to several thousands of units per ml can be used. Human leukocytes are then added to a concentration of about 3 - 15 million cells per mL, preferably 7 - 11 million cells per mL. The similar size of the enhancing effect observed using the different cell concentrations makes it probable that the effect can be seen also at cell concentrations outside this range. The priming step is normally allowed to proceed for 1 - 5 hours, preferably 1.5 - 2 hours.

Induction of interferon is performed by addition of Sendai virus, generally 30 mL virus from allantoic fluid per litre incubation medium but both smaller and larger amounts showed good inducing capacity and the most

preferred range is 500-2000 hemagglutinating virus particles per cell. A decrease of the incubation temperature to 29°C - 32 °C is performed (Morser, J. And Shuttleworth, J. **(1981)** *J. Gen. Virology* ***56,*** 163-174) after another 1 to 4 hours, preferable after 1 to 2 hours.

The optimal timing for adding the different enhancing agents varies, but in general an increase in α-interferon production is seen when the substances are added either at the same time as the cells or up to several hours after the induction, preferentially close to the time of the temperature decrease.

The concentration range where the additives are effective in increasing the interferon production varies between the different compounds but for the least toxic solvents a positive effect can be seen in the range of 1 mM - 0.3 M, preferably in the range of 3 mM - 50 mM and most preferably within 5 mM - 20mM. For the more toxic solvents the suitable concentration lies within the more limited ranges. For the theophylline, 2-amino-5-bromo-6-methyl-4-pyrimidinol and thymine the effective range is 5 μM - 0.5 mM, preferably 20 μM - 0.15 mM. After these additions, the incubation is allowed to proceed overnight with continuous stirring. A centrifugation step is performed to remove the cells and the supernatants are analysed by an ELISA to quantify α-interferon.

The invention will now be illustrated with the following non-limiting examples and with reference to the figures:

Figure legends

Figure 1A:

Effect on α-interferon production in Sendai virus induced human leukocytes by addition of different organic solvents to the incubation medium. The amount of α-interferon produced is calculated as percent of a reference with no addition of organic solvents (=100 %).

Figure 1B:

Effect on α-interferon production in Sendai virus induced human leukocytes by addition of different organic solvents in combination with 2-ABMP to the incubation medium. The amount of α-interferon produced is calculated as percent of a reference with no addition of enhancing agent (=100 %).

Figure 2:

Effect on α-interferon production in Sendai virus induced human leukocytes by addition of different purine and pyrimidine derivatives at different NMP concentrations. The amount of α-interferon produced is calculated as percent of a reference with no addition of enhancing agents (=100 %).

Figure 3:

Effect on α-interferon production in Sendai virus induced human leukocytes by addition of different amounts of theophylline at different DMSO concentrations. The amount

of α-interferon produced is calculated as percent of a reference with no addition of enhancing agents (=100 %).

Figure 4:

Effect on α-interferon production in Sendai virus induced human leukocytes by addition of NMP and theophylline to the incubation medium in laboratory scale fermentors. The amount of α-interferon produced is calculated as percent of a reference with no addition of enhancing agents (=100 %).

Table 1

Effect on α-interferon production of addition of enhancing agents, 2-ABMP and DMSO, both to Sendai virus induced human leukocytes and to non induced human leukocytes.

<u>Example 1</u>

α-interferon production by Sendai virus induced human leukocytes after incubation with various organic solvents.

Human leukocytes prepared from buffy coats and used in concentrations between 8 and 11 million cells per mL are incubated in a basal media (either EMEM or a modified EMEM) supplemented with 1.5 g/L of tricine and 4 % (v/v) PEG precipitated plasma. The experiments are performed in a volume of 40 mL medium in 100 mL glass bottles under continuous stirring. The cells are primed with 100 IU/mL α-interferon for 1.5 hours at 37°C before addition of 30 mL/L Sendai virus. After 1.5 hours the incubation temperature is decreased to 30°C and the organic solvents are added to the incubation medium. The solvent used are acetone, DMSO (final concentration: 5 mL/L), 2-butanone,

NN-dimethylacetamide, NMP, tetramethylene sulfoxide (2.5 mL/L), TMU (1.5 mL/L), 2-pyrrolidinone (1.25 mL/L), 4-metyl-2-pentanone, 1,3-dimetyl-2-imidazolidinone and N-ethyl-2-pyrrolidinone (0.6 mL/L). The incubation is then allowed to proceed overnight. The cells are removed by a centrifugation step and the supernatants are analysed by an ELISA to quantify α-interferon.

The results are shown in Figure 1A.

The above incubation conditions are used for the example shown in Figure 1B, but in this case some of the organic solvents are combined with 50 to 100 μg/mL of 2-ABMP.

<u>Example 2</u>

α-interferon production in Sendai virus induced human leukocytes after incubation with purine and pyrimidine derivatives, in the absence or presence of an organic solvent.

Example 1 is repeated with the same incubation conditions but with addition of one purine and two pyrimidine derivatives either alone or in combination with NMP. The purine used is theophylline (50 μg/ml) and the pyrimidines are 2-ABMP (50 μg/ml) or thymine (140 μg/ml).

The results are shown in Figure 2.

<u>Example 3</u>

α-interferon production in Sendai virus induced human leukocytes after incubation with theophylline and an organic solvent at varying concentrations.

The procedure from example 1 is repeated but with addition of the organic solvent DMSO and the purine theophylline. DMSO is added at 2.5 mL/L or 5 mL/L. The theophylline concentration varies between 5 μg/mL to 100 μg/mL.

The results are shown in Figure 3.

Example 4

α-interferon production in Sendai virus induced human leukocytes incubated in laboratory scale fermentors

An up-scaling is performed where the results obtained in the small scale is used to find suitable conditions for the laboratory fermentors. Different experiments are made to compare the α-interferon production in a fermentor where NMP and theophylline are added with a reference fermentor where no enhancing agent is added. The experiments are performed in laboratory scale fermentors using 2 L medium per vessel. The amount of theophylline is 50 μg/mL, while NMP varies from 1.75 mL/L to 3 mL/L. The incubation conditions are in accordance with Example 1. The results are shown in Figure 4.

Example 5

α-interferon production in human leukocytes incubated with 2-ABMP and DMSO. Effects of virus induction.

Example 1 is repeated with the same incubation conditions but with addition of 2-ABMP in combination with DMSO. The enhancing agents are added both to Sendai virus induced leukocytes and to non induced leukocytes and the effect on the α-interferon production is compared. The 2-ABMP is added at a concentration of 50 μg/ml and the amount of DMSO added is 5 mL/L.

The results are shown in Table 1.

Table 1

Viral induction	Enhancing agent	Yield of α-interferon
Sendai virus (30 mL/L)	None	100 %
Sendai virus (30 mL/L)	2-ABMP (50 μg/mL) and DMSO (5 mL/L)	146 %
None	2-ABMP (50 μg/mL) and DMSO (5 mL/L)	< 1 %

Conclusion

In the present invention it has been shown that addition of different organic solvents as well as different purines and pyrimidines reproducibly increases the production of α-interferon in virus induced human leukocytes. The largest increase is obtained when the purine or pyrimidine derivatives are combined with an organic solvent.

CLAIMS

1. A process for the production of α-interferon comprising the steps:

i) inducing of human leukocytes by means of a virus,

ii) treating the leukocytes with an enhancing agent selected from

a) xanthine, pyrimidinol, pyrimidinone, theophylline, theobromine, enprophylline, hypoxanthine, 8-phenyltheophylline, 2-amino-5-bromo-6-methylpyrimidinol, 2-amino-6-methyl-4-pyrimidinol and thymine;

b) an organic solvent selected from the group consisting of non-aromatic ketones, aliphatic or cyclic amides, alkylated aliphatic or cyclic urea derivatives and aliphatic or cyclic sulfoxides;

or a combination of the compounds from a) with an organic solvent from b).

2. A process according to claim 1, characterized in that the leukocytes are monocytes.

3. A process according to any one of claims 1 and 2, characterized in that the enhancing agent is added at the same time or up to 4 hours after the virus induction.

4. A process according to any one of claims 1 - 3, characterized in that the virus is Sendai virus.

5. A process according to any one of claims 1 - 4, characterized in that the enhancing agent is theophylline.

6. A process according to any one of claims 1 - 4, characterized in that the enhancing agent is 2-amino-5-bromo-6-methyl-4-pyrimidinol.

7. A process according to any one of claims 1 - 4, characterized in that the enhancing agent is thymine.

8. A process according to any one of the preceding claims, characterized in that the organic solvent is any of acetone, 2-butanone, 1,3-dimethyl-2-imidazolidinone, dimethylsulfoxide, N-ethyl-2-pyrrolidinone, 4-methyl-2-pentanone, N-methyl-2-pyrrolidinone, 2-pyrrolidinone, tetramethylene sulfoxide and N,N-dimethylacetamide.

9. A process according to claim 8, characterized in that the solvent is N-methyl-2-pyrrolidinone.

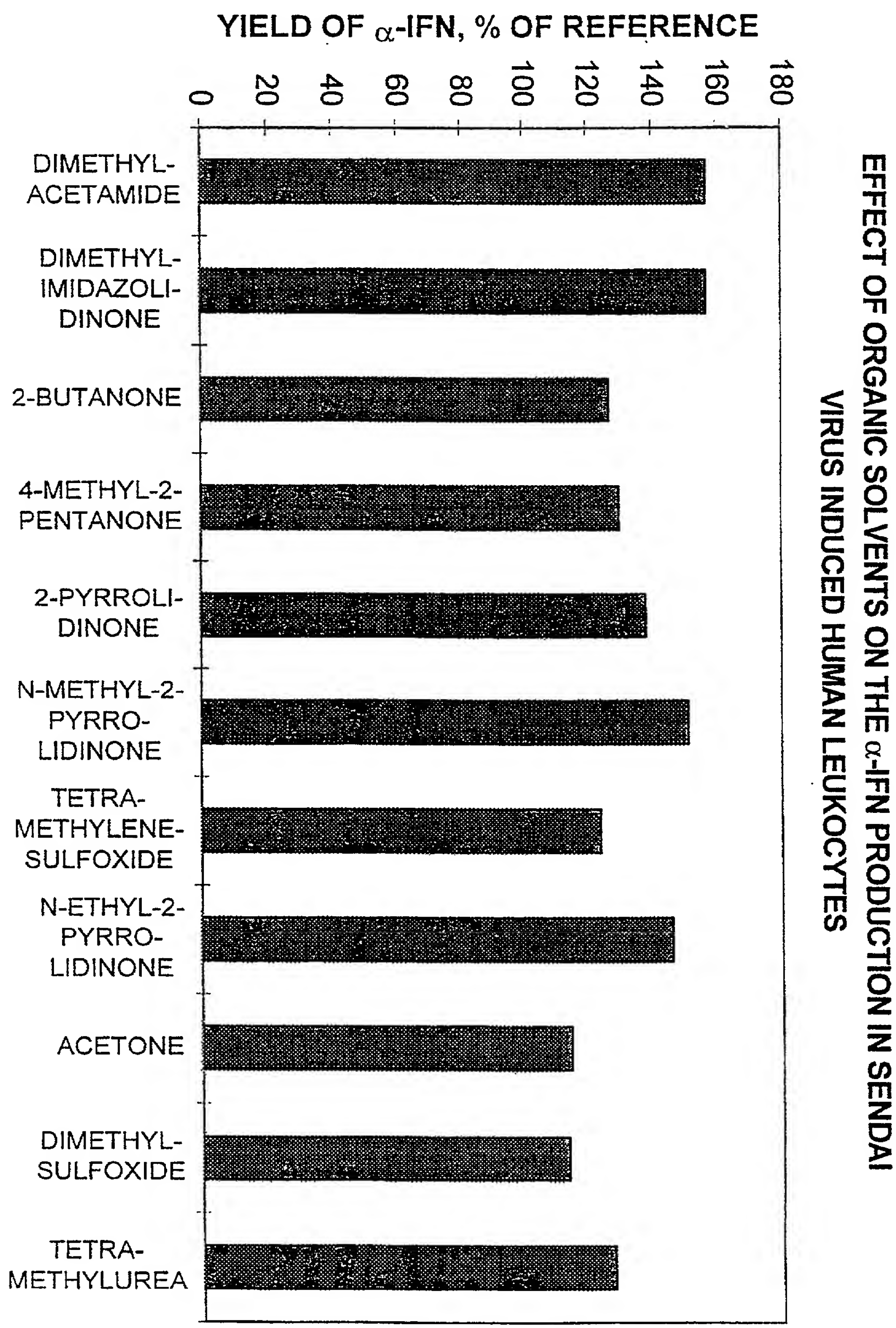
EFFECT OF ORGANIC SOLVENTS ON THE α-IFN PRODUCTION IN SENDAI VIRUS INDUCED HUMAN LEUKOCYTES
YIELD OF α-IFN, % OF REFERENCE
0
20
40
60
80
100
120
140
160
180
DIMETHYL-ACETAMIDE
DIMETHYL-IMIDAZOLI-DINONE
2-BUTANONE
4-METHYL-2-PENTANONE
2-PYRROLI-DINONE
N-METHYL-2-PYRRO-LIDINONE
TETRA-METHYLENE-SULFOXIDE
N-ETHYL-2-PYRRO-LIDINONE
ACETONE
DIMETHYL-SULFOXIDE
TETRA-METHYLUREA

FIGURE 1A

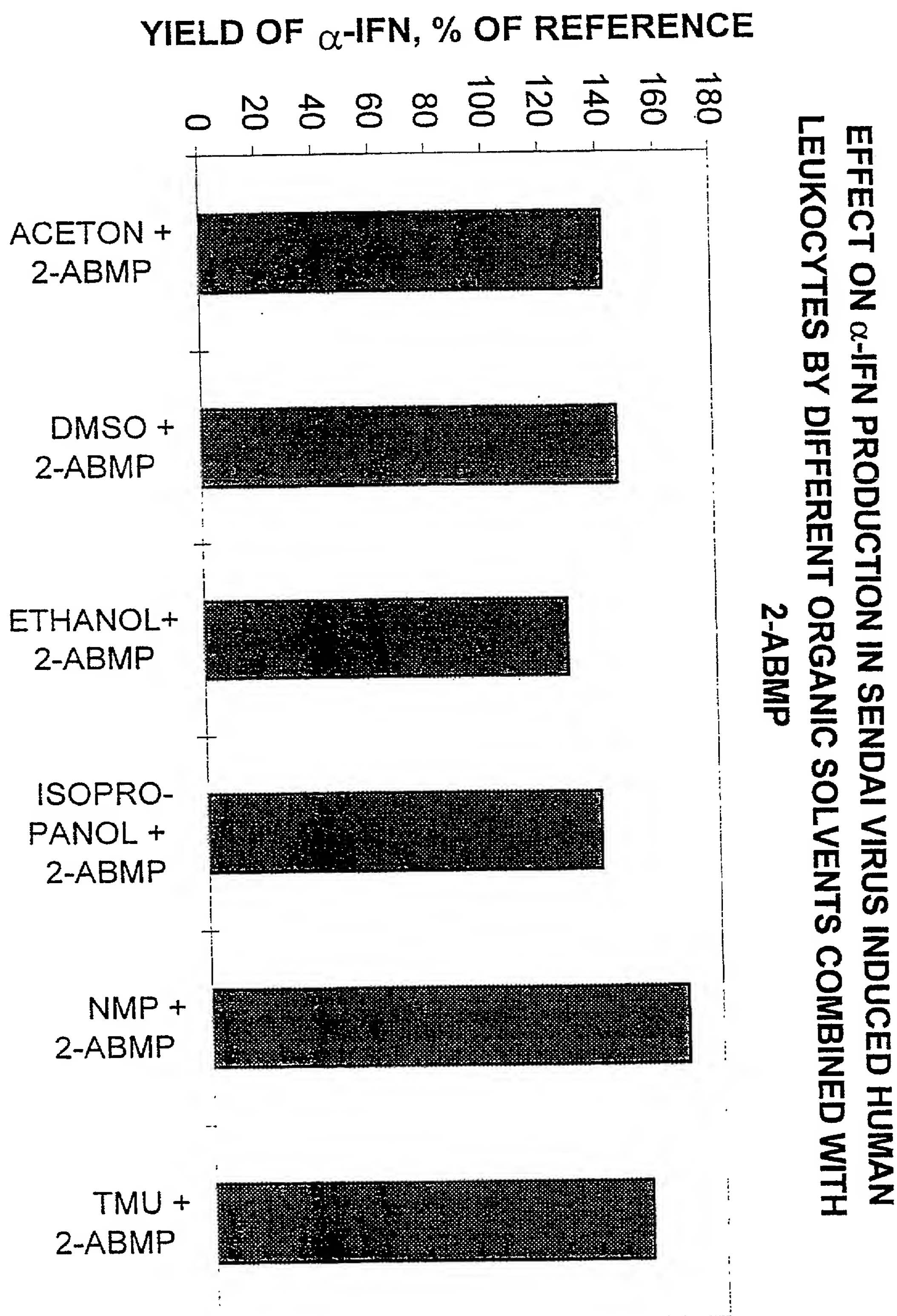
EFFECT ON α-IFN PRODUCTION IN SENDAI VIRUS INDUCED HUMAN LEUKOCYTES BY DIFFERENT ORGANIC SOLVENTS COMBINED WITH 2-ABMP
YIELD OF α-IFN, % OF REFERENCE
0
20
40
60
80
100
120
140
160
180
ACETON + 2-ABMP
DMSO + 2-ABMP
ETHANOL+ 2-ABMP
ISOPRO-PANOL + 2-ABMP
NMP + 2-ABMP
TMU + 2-ABMP

FIGURE 1B

09/869269

EFFECT ON IFN-TITER OF PURINE AND PYRIMIDINE DERIVATIVES AT DIFFERENT N-METHYL-2-PYRROLIDINONE (NMP) CONCENTRATIONS

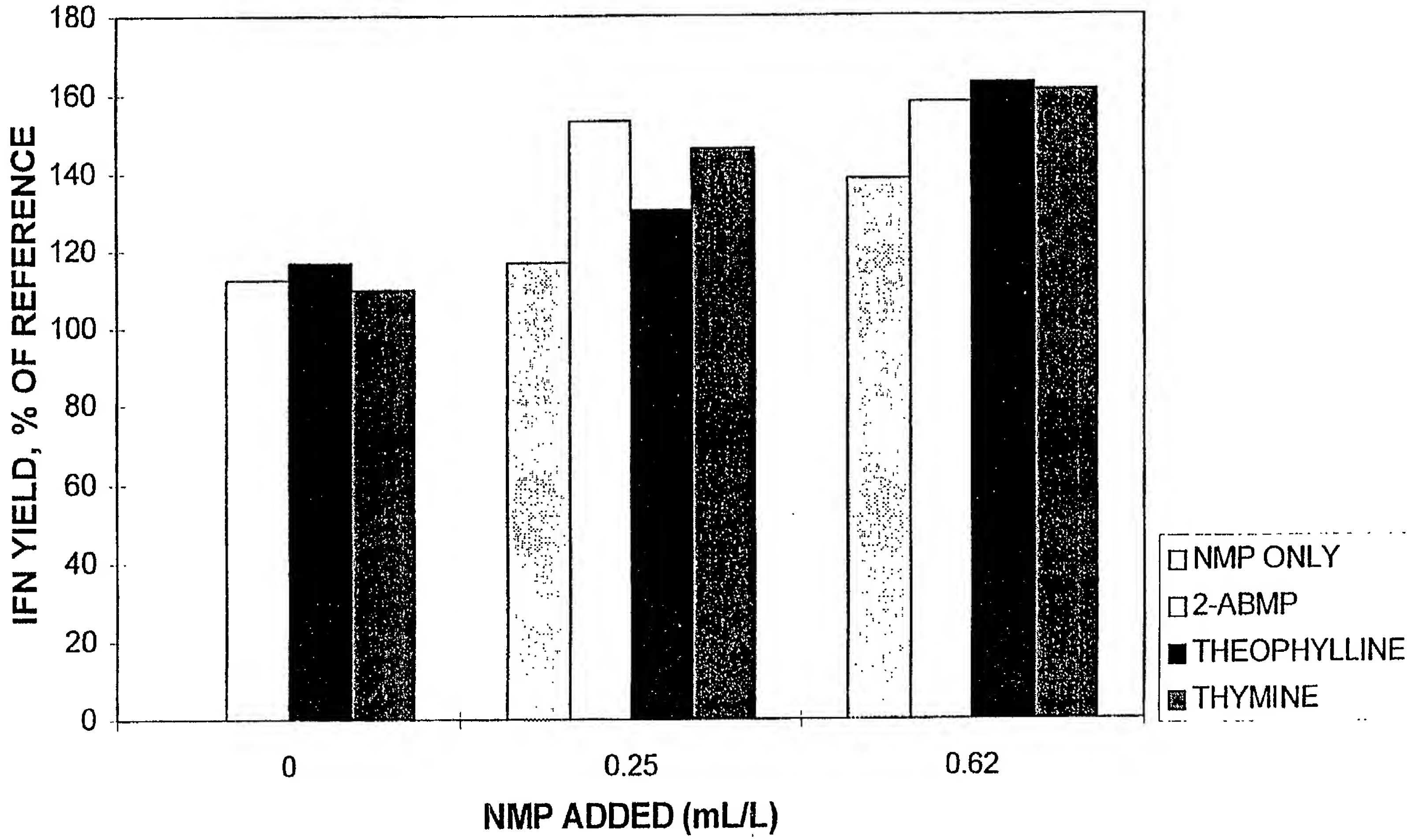

FIGURE 2

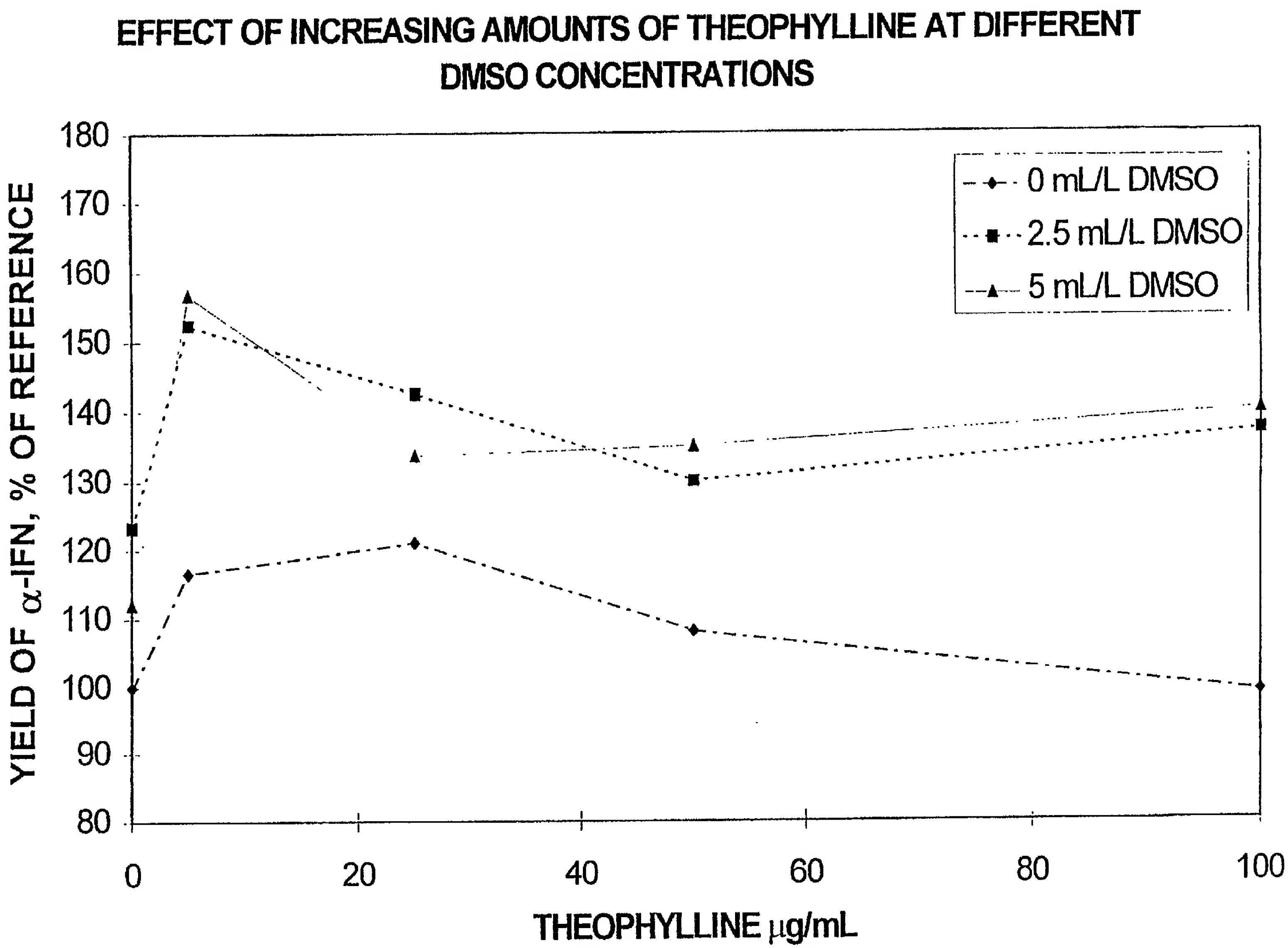
EFFECT OF INCREASING AMOUNTS OF THEOPHYLLINE AT DIFFERENT DMSO CONCENTRATIONS
YIELD OF α-IFN, % OF REFERENCE
180
170
160
150
140
130
120
110
100
90
80
0
20
40
60
80
100
THEOPHYLLINE μg/mL
0 mL/L DMSO
2.5 mL/L DMSO
5 mL/L DMSO

FIGURE 3

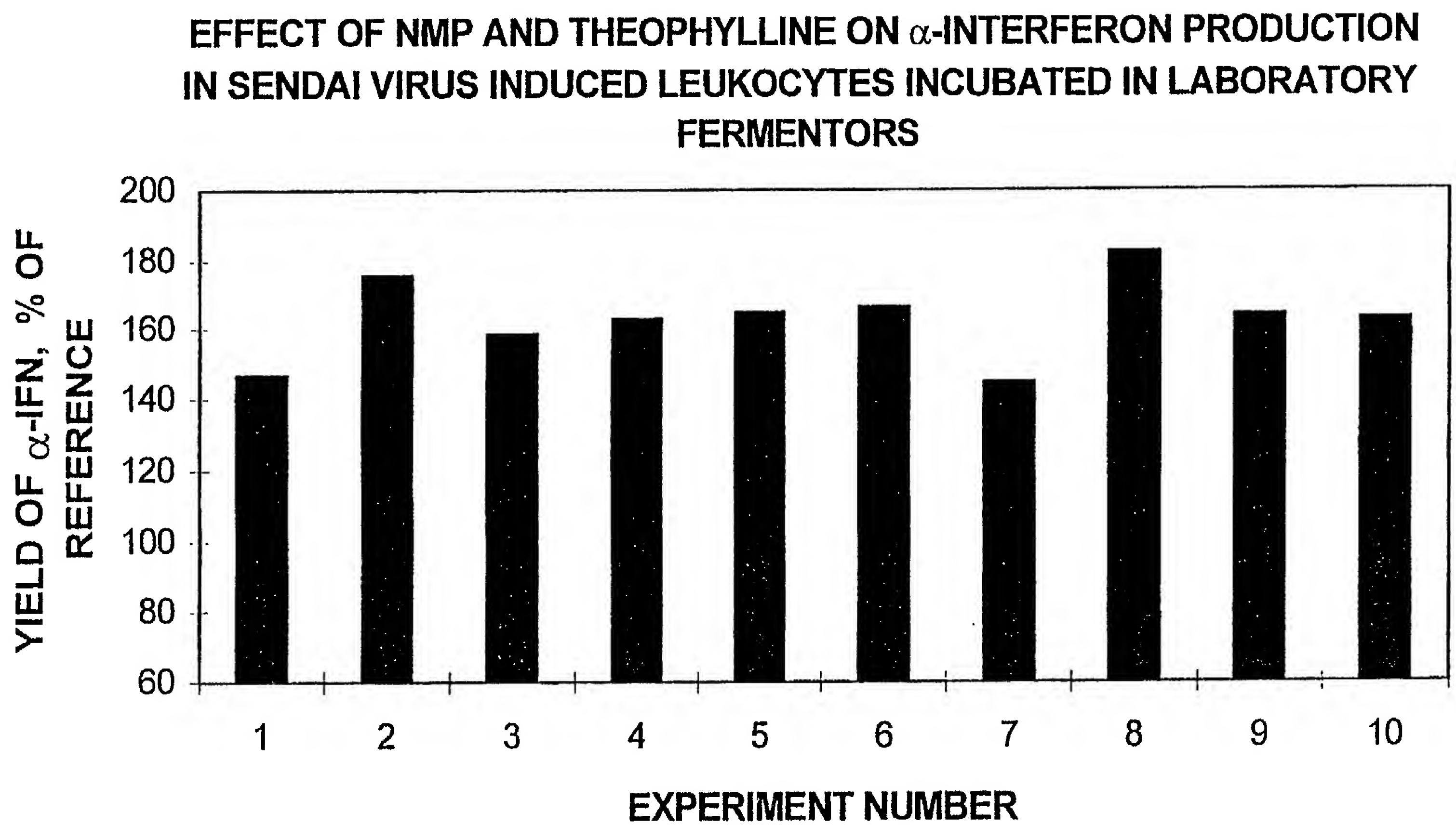
EFFECT OF NMP AND THEOPHYLLINE ON α-INTERFERON PRODUCTION IN SENDAI VIRUS INDUCED LEUKOCYTES INCUBATED IN LABORATORY FERMENTORS
YIELD OF α-IFN, % OF REFERENCE
200
180
160
140
120
100
80
60
1
2
3
4
5
6
7
8
9
10
EXPERIMENT NUMBER

FIGURE 4

COMBINED DECLARATION AND POWER OF ATTORNEY FOR UTILITY PATENT APPLICATION	Attorney's Docket No. 003300-790

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name;

I BELIEVE I AM THE ORIGINAL, FIRST AND SOLE INVENTOR (if only one name is listed below) OR AN ORIGINAL, FIRST AND JOINT INVENTOR (if more than one name is listed below) OF THE SUBJECT MATTER WHICH IS CLAIMED AND FOR WHICH A PATENT IS SOUGHT ON THE INVENTION ENTITLED:

MODIFICATION OF INTERFERON ALPHA PRODUCTION

the specification of which

(check one) ☐ is attached hereto;

☒ was filed on December 22, 1999 as

Application No. PCT/SE99/02446

and was amended on January 24, 2001 ;
(if applicable)

I HAVE REVIEWED AND UNDERSTAND THE CONTENTS OF THE ABOVE-IDENTIFIED SPECIFICATION, INCLUDING THE CLAIMS, AS AMENDED BY ANY AMENDMENT REFERRED TO ABOVE;

I ACKNOWLEDGE THE DUTY TO DISCLOSE TO THE OFFICE ALL INFORMATION KNOWN TO ME TO BE MATERIAL TO PATENTABILITY AS DEFINED IN TITLE 37, CODE OF FEDERAL REGULATIONS, Sec. 1.56 (as amended effective March 16, 1992);

I do not know and do not believe the said invention was ever known or used in the United States of America before my or our invention thereof, or patented or described in any printed publication in any country before my or our invention thereof or more than one year prior to said application; that said invention was not in public use or on sale in the United States of America more than one year prior to said application; that said invention has not been patented or made the subject of an inventor's certificate issued before the date of said application in any country foreign to the United States of America on any application filed by me or my legal representatives or assigns more than twelve months prior to said application;

I hereby claim foreign priority benefits under Title 35, United States Code Sec. 119 and/or Sec. 365 of any foreign application(s) for patent or inventor's certificate as indicated below and have also identified below any foreign application for patent or inventor's certificate on this invention having a filing date before that of the application(s) on which priority is claimed:

(05/01)

COMBINED DECLARATION AND POWER OF ATTORNEY	Attorney's Docket No. 003300-790

COUNTRY/INTERNATIONAL	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
Sweden	9804583-4	29 December 1998	YES X NO_
			YES_ NO_

I hereby appoint the following attorneys and agent(s) to prosecute said application and to transact all business in the Patent and Trademark Office connected therewith and to file, prosecute and to transact all business in connection with international applications directed to said invention:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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(05/01)